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(54) Title: APOLIPOPROTEIN E POLYMORPHISM AND ALZHEIMER'S DISEASE

(57) Abstract

The present invention relates to methods for the clinical determination of the risk of the late-onset of Alzheimer's disease of a patient or for the diagnosis or prognosis of Alzheimer's disease in a patient. The methods involve determining the number of copies of the apoE gene allele E4 in a biological sample of the patient; wherein one or two copies of E4 indicates that the patient is afflicted or at risk of developing Alzheimer's disease with a lowered age of death. Oligonucleotide primers specific to apoE2, apoE3 or apoE4 or primers specific to a region of said apoE gene common to apoE2, apoE3 and apoE4 alleles are used to determine the number of copies of the apoE4 isoform.

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APOLIPOPROTEIN E POLYMORPHISM AND ALZHEIMER'S DISEASE

BACKGROUND OF THE INVENTION

Apolipoprotein E (apoE) is a well characterized lipophilic protein associated with plasma and cerebrospinal fluid lipoproteins. ApoE is synthesized primarily by the liver, but also at other sites including brain, macrophages and adrenals (Elshourbagy N.A. et al., Proc. Natl. Acad. Sci., 1985, 82: 203-207). Furthermore, apoE is unique among apolipoproteins in that it is has a special relevance to the central and peripheral nervous systems. Apolipoprotein E (apoE) is important in modulating cholesterol and phospholipid transport from one cell to another. It is a key determinant in the cellular recognition and internalization of cholesterol-rich lipoproteins in the developing brain and in the response to neuronal injury (Poirier J. et al., Mol. Brain Res., 1991, 11: 97-106; Poirier J. et al., Neuroscience, 1993, 55: 81-90). It also plays a fundamental role in the central nervous system (CNS) during synaptic remodelling induced by neuronal differentiation (Poirier J. et al., Mol. Brain Res., 1991, 11: 97-106; Poirier J. et al., Neuroscience, 1993, 55: 81-90; Poirier J. et al., Mol. Brain Res., 1991, 9: 191-195).

The apoE gene is located on chromosome 19, within a region which had previously been associated with familial late-onset cases of Alzheimer's disease (Schellenberg G.D. et al., Ann. Neurol., 1992, 31: 223-227). The structural gene for apoE is polymorphic, the three most common isoforms coded for are designated E2, E3 and E4 as illustrated in Fig. 1. These isoforms differ by amino acid substitutions at one or both of two sites, residues 112 and 158. The E2 isoform has Cys residues at sites 112 and 158. The E3

isoform has a Cys residue at site 112 and an Arg at site 158, and the E4 isoform has an Arg residue at sites 112 and 158. An individual thus may be homozygous (E2/2, E3/3 or E4/4) or heterozygous (E4/2, E4/3 or E3/2).

ApoE has been shown to be present in plaques and dystrophic neurites that characterize the neuropil of the Alzheimer's brain (Namba Y. et al., Brain Res., 1991, 541: 163-166; Wisniewski T. et al., Neurosci. Lett., 1991, 135: 235-238). Although apoE mRNA and protein content is increased in response to neuronal cell loss in rat (Poirier J. et al., Mol. Brain Res., 1991, 11: 97-106; Poirier J. et al., Neuroscience, 1993, 55: 81-90) and during demylination in humans with multiple sclerosis (Rifai N. et al., Clin. Chem., 1987, 33:1155-1157), apoE expression remains relatively unchanged in the hippocampus of Alzheimer's (AD) patients (Poirier J. et al., Basic, Clinical and Therapeutical Aspects of Alzheimer's and Parkinson's Diseases, 1990, Volume 1:191-195) despite marked neuronal cell loss and differentiation in this structure (Van Hoesen G.W. et al., Hippocampus, 1991, 1:1-8). Recent analysis of the apoE polymorphism in sporadic cases of Alzheimer's disease (Poirier J. et al., Lancet, 1993, 342: 697-699; Saunders A.M. et al., Neurology, 1993, 43:1467-1472) has shown an increased frequency of the apoE4 allele in these individuals. Moreover, there is a correlation between the age of onset for the disease and the number of copies of the E4 allele in that E4 homozygotes were shown to have an earlier age of onset (Poirier J. et al., Lancet, 1993, 342: 697-699). Increased frequencies of the E4 allele have also been reported in familial cases of late onset Alzheimer's disease (Corder E.H. et al., Science, 1993, 261:921-923).

Although the concept of subtypes of Alzheimer's disease remains controversial to date, it is clear that a significant portion of the Alzheimer's disease population shows extrapyramidal symptoms and neuropathological changes consistent with idiopathic Parkinson's disease (PD) (Boller F. et al., Ann. Neurol., 1980, 7:329-335; Ditter S.M. et al., Neurology, 1987, 37:754-760). The estimated prevalence of dementia in PD varies from 10% to 40% (Mayeux R. et al., Arch. Neurol., 1988: 45:260-262), the combination of Alzheimer's disease/Parkinson's disease (AD/PD) pathological features being common.

The present study was designed a) to examine the apoE4 allele frequency in definitive cases of sporadic Alzheimer's disease and, in control brains showing plaque and tangle counts below the consensus Alzheimer's disease threshold; b) to determine if the apoE genotype distribution varies in Alzheimer's disease (AD), Alzheimer's disease/Parkinson's disease (AD/PD) and Parkinson's disease (PD).

It would be highly desirable to be provided with a method for the clinical determination of the risk for the late-onset of Alzheimer's disease of a patient.

It would be highly desirable to be provided with a method to determine the number of copies of the apoE gene allele E4 in a biological sample of a patient to indicate the probable age of onset of Alzheimer's disease and the age of death.

SUMMARY OF THE INVENTION

One aim of the present invention is to have a classification of Alzheimer's disease for use in diagnostic, prognosis and selection of an appropriate treatment for the patient.

Another aim of the present invention is to have a method to determine the number of copies of the apoE gene allele E4 in a biological sample of a patient to indicate the probable age of onset of Alzheimer's disease.

In accordance with the present invention there is provided a method for the clinical determination of the risk for the late-onset of Alzheimer's disease of a patient or for the diagnosis or prognosis of Alzheimer's disease in a patient, which comprises the steps of:

a) amplifying genomic DNA encoding apoE in a biological sample of the patient using oligonucleotide primers specific to E2, E3 or E4; and

b) determining the number of copies of the apoE gene allele E4 in the biological sample wherein one or two copies of E4 indicates a level of incidence of late-onset of Alzheimer's disease and a lowered age of death.

Such specific apoE2, apoE3 and apoE4 primers, selected from the group consisting of:

D= 5' TACTGCACCAGGCGGCCTCG 3';

E= 5' TACTGCACCAGGCGGCCTCA 3';

F= 5' GCCTGGTACACTGCCAGTCG 3';

G= 5' GCCTGGTACACTGCCAGTCA 3'; and

H= 5' AAGGAGATTGAAGGCCTACAAAT 3',

are used in accordance with the present invention to amplify DNA segments of the apolipoprotein E2, E3 and E4 gene alleles using polymerase chain reaction (PCR) or ligase chain reaction (LCR).

Determination of the apoE4 gene copy number in a specific patient's sample is assessed by a) the utilization of gene specific DNA oligonucleotide primer sequences (described in details below) during PCR or LCR amplification that will amplify selectively

the apoE4 mutation or by b) direct DNA sequencing of the mutated apoE4 gene area.

Also in accordance with the present invention there is provided a method for the clinical determination of the risk for the late-onset of Alzheimer's disease of a patient or for the diagnosis or prognosis of Alzheimer's disease in a patient, which comprises the steps of:

a) amplifying genomic DNA encoding apoE in a biological sample of the patient using oligonucleotide primers specific to a region of the apoE gene common to apoE2, apoE3 and apoE4 alleles; and

b) genotyping the patient's apoE isoforms by DNA sequencing of the amplified DNA of step a) for indirectly determining the number of copies of the apoE gene allele E4 in the biological sample; wherein one or two copies of E4 indicates a level of incidence of late-onset of Alzheimer's disease and a lowered age of death.

Such apoE primers are selected from the sets consisting of:

a) 5' ACAGAATTCGCCCCGGCCTGGTACAC 3';
5' TAAGCTTGGCACGGCTGTCCAAGGA 3'; and

b) 5' ATCAAGCTTTCGCCCGCCCATCCCAGCCCTTC 3';
5' CGTGAATTCGCATGGGCTGCAGGCTTCGGCGTTC 3'.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates three isoforms of apoE which are designated E2, E3 and E4;

Fig.2 shows a distribution of E4 allele frequency by age in post-mortem Alzheimer's diseases and controls;

Fig. 3 shows a distribution of E4 allele frequency by age in post-mortem Alzheimer's diseases and controls; and

Fig. 4 shows a distribution of E4 allele frequency by age in clinical (phenotype) and post-mortem (genotype) cases of Alzheimer's disease.

DETAILED DESCRIPTION OF THE INVENTION

Apolipoprotein E (apoE) is associated with Alzheimer's neurofibrillary tangles and β-amyloid protein in senile plaques. It also plays a critical role in the redistribution of lipids following differentiation and degeneration in the brain. Recent studies have shown high frequencies of the apoE4 allele in familial and sporadic cases of Alzheimer's disease (AD).

In accordance with the present invention, the apoE genotype was determined by allele-specific extension of 113 post-mortem cases of sporadic Alzheimer's disease and 77 age-matched control brains shown to be free of Alzheimer's disease neuropathological feature, and then calculated the frequency of the various allelic forms of apoE (E2, E3, E4; Fig. 1). Because Alzheimer's disease and Parkinson's disease (PD) share several neuropathological characteristics, the apoE genotypes were also determined in seventeen (17) post-mortem cases of idiopathic Parkinson's disease and nineteen (19) cases with both Alzheimer's and Parkinson's diseases.

The overall frequency of the E4 allele in the Alzheimer's disease cases was 33% compared to 5% in controls while that of E2 was 2% in Alzheimer's disease versus 5% in controls and that of E3, 65% in Alzheimer's disease versus 90% in controls ($p<0.001$). Of all the individuals examined in this study, 91%

(10/11) of those with two (2) copies of the E4 allele, and 90% (55/61) of those with one (1) copy of the E4 allele were neuropathologically confirmed cases of Alzheimer's disease. The frequencies of the E4 and E3 alleles were similar for AD and AD/PD whereas the E4 allele was absent in PD without dementia.

A single copy of the E4 allele is sufficient to represent a major biological risk factor in sporadic Alzheimer's disease.

Brain Tissue Samples

Neuropathologically confirmed brains were obtained from the Douglas Hospital Brain Bank (Montréal, Qc, Canada). Seventy seven controls (C) representing a random population and a hundred and thirteen (113) sporadic Alzheimer's disease frozen brains were used in this study. Seventeen were Parkinson's disease without dementia and nineteen (19) with AD/PD. Neuropathological confirmation of idiopathic Parkinson's disease was based on the presence of Lewy Bodies and on the loss of pigmented neurons in the *substantia nigra pars compacta* as described in Aubert et al. (Aubert I., et al., J. Neurochem., 1992, 58:529-541).

The diagnosis of Alzheimer's disease was confirmed neuropathologically in all cases according to the criteria of Khachaturian et al. (Khachaturian Z.S., Arch. Neurol., 1985, 42:1097-1105) as adapted by Aubert et al. (Aubert I. et al., J. Neurochem., 1992, 58:529-541); all other conditions were excluded.

I- ApoE genotype using apoE2, apoE3 and apoE4 specific primers

High molecular weight DNA was isolated from frozen cerebellum or temporal cortex as adapted from

the procedure of Goelz et al. (Goelz S.E. et al., Biochem. Biophys. Res. Comm., 1985, 130: 118-126).

ApoE genotype was determined by allele-specific extension of purified brain DNA using a modification of the method of Main et al. (Main R.F. et al., J. Lipid Res., 1991, 32:183-187).

The primers labeled D, E, F, G, and H are synthesized using a Beckman™ DNA synthesizer. The primer sequences are as follows:

D= 5' TACTGCACCAGGCGGCCTCG 3';

E= 5' TACTGCACCAGGCGGCCTCA 3';

F= 5' GCCTGGTACACTGCCAGTCG 3';

G= 5' GCCTGGTACACTGCCAGTCA 3'; and

H= 5' AAGGAGATTGAAGGCCTACAAAT 3'.

Reactions were carried out in a volume of 50 uL containing 1 µg of DNA; deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxythymidine triphosphate and deoxyguanosine triphosphate, each 0.2 mmol/L; 10% dimethyl sulfoxide; 12.5 pmol of either primer D, E, F, G; 25 pmol of primer H; and 10 µL of 10 PCR reaction buffer (Vector Biosystem, Toronto, Ontario, Canada)

The DNA in the reaction mixture was first denatured for 10 min. at 96°C and then cooled to 4°C. One unit of Taq polymerase (Vector Biosystem, Toronto, Ontario, Canada) was then added to each sample. Each sample was reheated for 2 min. at 96°C and subjected to 30 cycles in a thermal cycler with each cycle consisting of a 10 sec denaturation at 96°C, 30 sec annealing at 58°C and 1 min. extension at 65°C. The reaction products were visualized by electrophoresis of 10 uL of the reaction mixture in a 1% agarose gel containing TPE (Tris phosphate EDTA) buffer (0.08 mol/L Tris-phosphate, 0.002 mol/L EDTA (ethylene diamine tetra-acetic acid) and ethidium bromide

(0.15 ug/mL) for 1 hr at 67°C. The gel were then photographed and the banding profile was compared to known standards.

RESULTS

The ages of controls were matched with the patients' ages (Table 1).

Table 1

ApoE Genotype and Allele Frequency

	ALZHEIMER'S DISEASE			CONTROLS			Eastern Canadian (n=102)
	All (n=113)	M (n=59)	F (n=54)	All(n=77)	M (n=53)	F (n=24)	
Age (yr)	76.4 (9.3)	75.9 (9.7)	76.9 (9.0)	70.1(15.2)	70.1(15.2)	70.1(15.2)	36.3 (8.5)
Genotype	**(%)**						
E4/4	8.8@	10.2	7.4	1.3	0.0	4.0	3.9
E3/3	40.7@	47.5	33.3	81.8	84.9	76.0&	61.7
E2/2	0.9@	1.7	0.0	0.0	0.0	0.0	2
E4/3	46.9@	37.3	57.4	6.5	3.8	12.0*	20.6
E4/2	1.7@	1.7	1.9	0.0	0.0	0.0	9.8
E3/2	0.9@	1.7	0.0	10.4	11.3	8.0*	2
Allele	**(frequency)**						
E4	0.33@	0.30	0.37	0.05	0.02	0.1*	0.152
E3	0.65@	0.67	0.62	0.90	0.92	0.86	0.77
E2	0.02@	0.03	0.01	0.05	0.06	0.04	0.078

Mean (SD) for age (yr);
@ $p<0.001$ versus controls;
& $p<0.05$ and * $p<0.001$ versus men;
Normolipidemic population from Eastern Canada.

The E3/3 genotype yielding a 145 base pair product with primer E, indicating the presence of Cys 112, and a 277 base pair product with primer F indicating the presence of Arg 158. The heterozygote E3/4 produces appropriately sized products with primers E and F identifying the E3 allele, but also produces a

145 base pair product with primer D indicating Arg 112. Homozygous E4/4 subjects react only with primers D and F marking the presence of Arg 112 and Arg 158, while E2/2 homozygotes react only with primers E and G indicating Cys 112 and Cys 158. In all reactions a 500 base pair fragment was observed indicating a successful amplification of the internal control.

The apoE genotype distribution and allele frequencies are given in Table 1. The distribution of apoE phenotypes among a random population from Eastern Canada as a comparison to the distribution of apoE genotypes was determined (Sing C.F. et al., Am. J. Hum. Genet., 1985, 37:268-278). The E4 allele frequency is significantly higher (6-fold) in the Alzheimer's population. The genotypic distribution profile shows an enrichment of the E4/4 and E4/3 genotypes and a marked reduction of the E3/3 genotype in AD. ApoE4/3 genotype is markedly enriched in women versus men, in both AD and control cases. Of all the individuals examined in this study, 91% (10/11) of those with 2 copies of the E4 allele, and 90% (55/61) of those with one copy of the E4 allele were definitive cases of AD. The neuropathological reports of the control individuals carrying one or two copies of the E4 allele indicates the presence of plaques and tangles below the AD threshold, but moderate to severe neuronal cell losses in Ammon's horn and subicular areas of the hippocampus.

Fig. 2 shows the distribution of E4 allele frequency as a function of age in post-mortem tissues of AD and control individuals. An extended analysis of the genotype distribution in AD, AD/PD and PD in Fig. 3 indicates that the frequency of the E4 and E3 alleles were similar in the AD and AD/PD groups whereas E4 allele was absent in PD.

The postmortem distribution of the E4 allele as a function of age in sporadic Alzheimer's disease is nearly identical the phenotypic distribution reported recently in clinically diagnosed Alzheimer's patients (Fig. 4).

In previous clinical study up to 20% of the non-demented controls had at least one copy of the E4 allele and was hypothesized that a large proportion of the individuals would develop AD with time (Poirier J. et al., Lancet, 1993, 342: 697-699). Due to the complexity and logistical problems associated with a 10 to 15 year longitudinal study of the non-demented E4 controls, the alternative strategy of the case-control study was described in accordance with the present invention. To examine the relationship between the E4 allele and the incidence of AD, genotypic analysis of apoE was carried out on samples of autopsied brains from individuals (age 43 to 95 years) with little or no neurodegenerative changes associated with AD.

The present results demonstrate a strong association between the E4 allele and neuropathologically confirmed sporadic Alzheimer's disease. The overall frequency of the E4 allele in the AD cases was 33% compared to 5% in controls while that of E2 was 2% in AD *versus* 5% in controls and that of E3, 65% in AD *versus* 90% in controls. These frequencies are similar to those reported previously in sporadic AD and late onset familial AD (Poirier J. et al., Lancet, 1993, 342: 697-699; Corder E.H. et al., Science, 1993, 261:921-923). Furthermore, comparison of E4 allele frequency as a function of age in clinical and autopsied cases of sporadic AD shows a very similar distribution profile (Poirier J. et al., Lancet, 1993, 342: 697-699),

supporting the diagnostic value of apoE phenotyping in living patients.

The present genotyping study has also revealed that 91% (10/11) of E4 homozygotes and 90% (55/61) of E4 heterozygotes had definitive (neuropathologically confirmed) Alzheimer's disease. Thus, the presence of a single copy of the E4 allele represents a major biological risk factor for sporadic AD. Less than 10% of controls (n=77) carried the E4 allele. Although all controls had plaque and tangle counts below the AD threshold level, the E4 allele carriers (1 homozygotes and 5 heterozygotes) demonstrated moderate to severe neuronal loss in the hippocampal Ammon's horn and subicular areas. Presumably, these individuals might represent an early phase (pre-symptomatic) of AD pathophysiology. If this assumption proves to be correct, the results of the present invention would clearly indicate that neuronal cell loss in the hippocampal area of E4 carriers precedes the formation of plaques and tangles.

Epidemiological studies have shown that risk of dementia in subjects suffering from Parkinson's disease is twice that of healthy controls: the overall cumulative probability of developing dementia in a period of 5 years is 21% in PD versus 5.7% in healthy controls (Ditter S.M. et al., Neurology, 1987, 37:754-760). A common pathophysiology has been suggested by histopathological and neurochemical observations: the presence in cortical areas of demented parkinsonians of plaques, tangles, neuronal cell losses (Boller F. et al., Ann. Neurol., 1980, 7:329-335; Ditter S.M. et al., Neurology, 1987, 37:754-760) and the concomitant loss of cholinergic and serotoninergic activities (Aubert I. et al., J. Neurochem., 1992, 58:529-541; D'Amato R.J. et al., Ann. Neurol., 1987, 22:229-238). The present results

do not support the concept of a common pathophysiology in AD and PD. The distribution of apoE isoforms as determined by genotypic analysis in AD, AD/PD and PD highlights two distinct entities: the PD population in which E4 is virtually absent and the AD population which is highly enriched in E4. The remaining patients showing neuropathological changes consistent with both AD and PD demonstrated an apoE isoform distribution that was very similar to AD, suggesting that the occurrence of PD in AD patients is an independent phenomenon in E4 carriers.

The immunological detection of apoE in plaques and tangles in AD and the enrichment of the E4 allele in AD suggest a fundamental role for apoE4 in the etiopathology of this disease. ApoE expression has been shown to be critical to the synaptic remodelling occurring in response to cell loss and differentiation in the CNS (Poirier J. et al., Mol. Brain Res., 1991, 11: 97-106; Poirier J. et al., Neuroscience, 1993, 55: 81-90; Poirier J. et al., Mol. Brain Res., 1991, 9: 191-195). In the normal aging human brain, the age-related decline in lipid levels and in cell number is apparently compensated by the active remodelling of neuronal pathways in an attempt to preserve the functional integrity of the CNS. Since synaptic and dendritic remodelling of neurons requires the induction of apoE and apoE/apoB low density lipoprotein (LDL) receptor expression (Poirier J. et al., Neuroscience, 1993, 55: 81-90), it is conceivable that the presence of apoE4 in the CNS may perturb cholesterol and phospholipid homoeostasis and interfere directly with synaptic plasticity. This could explain the marked decrease in presynaptic terminal density reported in cerebral cortex of AD, the synaptic losses in frontal and temporal cortices (Masliah E. et al., Neurosci. Lett.;

1989, 103: 234-239; Davies C.A. et al., J. Neurol. Sci., 1987, 78: 151-164) and the poor synaptic plasticity reported in the hippocampus of AD (deRuiter J.P. et al., Brain Res., 1987, 402: 217-229; Ransmayr G. et al., Neuroscience, 1989, 32: 701-714; Represa A. et al., Brain Res., 1988, 457: 355-359; Flood D.G. et al., Can. J. Neurol. Sci., 1986, 13: 475-479; Honer W.G. et al., Neurobiol. Aging, 1992, 13: 375-382).

In accordance with the present invention, the usefulness of apoE4 genotyping (or phenotyping) as a tool in either making the diagnosis of Alzheimer's disease or in identifying individuals at increased risk of having Alzheimer's disease was assessed. The sensitivity and specificity of the test was calculated using apoE4 carrier frequency in Alzheimer's disease and control subjects as reported herein based on neuropathologically confirmed cases. The results are set forth in Tables 2, 3 and 4 below.

Table 2

Gender-specific apolipoprotein E4 carrier frequency and derived test and population characteristics

AD cases	Controls	Sensitivity	Specificity	Odds Ratio (95% CI*)	Population Attribuable Risk
Men					
0.49	0.04	0.49	0.96	24.7 (5.49-111)	0.47
Women					
0.66	0.16	0.67	0.83	10.0	0.60
Combined					
0.57	0.07	0.57	0.92	15.5 (6.2-38-5)	0.53

CI*= confidence interval

Table 3

Overall ApoE4/4 frequency and derived test and population characteristics

AD cases	Controls	Sensitivity	Specificity	Odds Ratio (95% CI*)	Population Attribuable Risk
0.08	0.01	0.08	0.99	6.6 (0.81-53.0)	0.07

CI*= confidence interval

Table 4

Age of death in Alzheimer's disease cases

ApoE genotype	Mean Age S.E.M	**
3/3	78.09	1.45
4/3	76.58	0.93
4/4	72.10	3.04*

*p<0.049657 : 3/3 versus 4/4

Table 5

Incidence of late-onset Alzheimer's disease

2 copies of E4= >90%
1 copy of E4= 70%
0 copy of E4= impossible to determine with precision

The analysis of the apoE4 allele copy number (0, 1 or 2 copies) reveals a statistically significant difference in the age of death in Alzheimer's disease subjects homozygous for apoE3 and apoE4.

This is perfectly consistent with our previously published clinical data on the same Eastern Canadian population, where the age of onset for Alzheimer's disease is apoE4 allele copy number dependent. in other words, as apoE4 gene dose increase from 0, 1 to 2, the mean age of death for

Alzheimer's disease decreases from 78 years (o copy), 76.5 (1 copy) to 72.1 (2 copies). The apoE4 allele copy numbers, in addition to allowing us to determine the extent of the risk for having Alzheimer's disease, allows us to predict the age of death of someone for which we determine the apoE genotype and the presence of clinical features consistent with a diagnostic of probable Alzheimer's disease.

II- ApoE genotype by DNA sequencing

Enzymatic amplification of genomic DNA

Genomic DNA is extracted from whole blood or frozen tissue and 1-5 ug of genomic DNA is used directly for amplification of the apoE genomic DNA. Set of primers such as:

a) 5' ACAGAATTCGCCCCGGCCTGGTACAC 3'
   5' TAAGCTTGGCACGGCTGTCCAAGGA 3', and

b) 5' ATCAAGCTTTCGCCCGCCCATCCCAGCCCTTC 3'
   5' CGTGAATTCGCATGGGCTGCAGGCTTCGGCGTTC 3'

are used to amplify 245 base pair fragment that contains amino acid residue 112 and 158 for distinguishing common apoE2, apoE3 and apoE4 alleles.

The amplified DNA is subsequently purified.

Automated DNA sequencing

Automated DNA sequence analysis is based on fluorescence detection of DNA fragments extended from fluorescent dye-linked primers by the deoxy-chain terminator method of Sanger et al. (Proc. Natl. Acad. Sci., 1977, 74: 5463-5467). A polymerase (T4 or Klewnow) is used together with purified nucleotides to extend the bound DNA primers. Amplified fluorescent fragments are then run on a 8 M urea-6% polyacrylamide gel and fragments migration profiles are monitored on an automated DNA sequencer such as ALF™ sequencer (Pharmacia Corp.). The genomic DNA is preferably amplified using the set

a) of primers, whereas the set b) of primers is used with fluorescent dyes for the sequencing. Data points are collected on a computer and the nucleotide sequence of apoE isoform is determined and the number of apoE4 is then indirectly assessed.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

I CLAIM:

1. A method for the clinical determination of the risk for the late-onset of Alzheimer's disease of a patient, which comprises:

a) amplifying genomic DNA encoding apoE in a biological sample of said patient using oligonucleotide primers specific to E2, E3 or E4; and

b) determining the number of copies of the apoE gene allele E4 in said biological sample wherein one or two copies of E4 indicates a level of incidence of late-onset of Alzheimer's disease and a lowered age of death.

2. The method of claim 1, wherein said apoE specific primers are selected from the group consisting of:

D= 5' TACTGCACCAGGCGGCCTCG 3';
E= 5' TACTGCACCAGGCGGCCTCA 3';
F= 5' GCCTGGTACACTGCCAGTCG 3';
G= 5' GCCTGGTACACTGCCAGTCA 3'; and
H= 5' AAGGAGATTGAAGGCCTACAAAT 3'.

3. The method of claim 1, wherein said amplifying step a) is effected by polymerase chain reaction technique or by ligase chain reaction technique.

4. The method of claim 3, wherein said determining step b) is effected by gel electrophoresis of said amplified DNA of step a).

5. The method of claim 1, wherein one copy of apoE4 indicates that said patient has about 70% risk of developing Alzheimer's disease and a mean age of death of about 76.5 years.

6. The method of claim 1, wherein two copies of apoE4 indicates that said patient has about 90% risk of developing Alzheimer's disease and a mean age of death of about 72.1 years.

7. A method of diagnosing or prognosing Alzheimer's disease in a patient, which comprises:

a) amplifying genomic DNA encoding apoE in a biological sample of said patient using oligonucleotide primers specific to E2, E3 or E4; and

b) determining the number of copies of the apoE gene allele E4 in said biological sample wherein one or two copies of E4 indicates that the patient is afflicted or at risk of developing Alzheimer's disease with a lowered age of death.

8. The method of claim 7, wherein said apoE specific primers are selected from the group consisting of:

D= 5' TACTGCACCAGGCGGCCTCG 3';
E= 5' TACTGCACCAGGCGGCCTCA 3';
F= 5' GCCTGGTACACTGCCAGTCG 3';
G= 5' GCCTGGTACACTGCCAGTCA 3'; and
H= 5' AAGGAGATTGAAGGCCTACAAAT 3'.

9. The method of claim 7, wherein said amplifying step a) is effected by polymerase chain reaction technique or by ligase chain reaction technique.

10. The method of claim 9, wherein said determining step b) is effected by gel electrophoresis of said amplified DNA of step a).

11. The method of claim 7, wherein one copy of apoE4 indicates that said patient has about 70% risk of developing Alzheimer's disease and a mean age of death of about 76.5 years.

12. The method of claim 7, wherein two copies of apoE4 indicates that said patient has about 90% risk of developing Alzheimer's disease and a mean age of death of about 72.1 years.

13. A method for the clinical determination of the risk for the late-onset of Alzheimer's disease of a patient by genotyping said patient's apoE isoforms, which comprises:

a) amplifying genomic DNA encoding apoE in a biological sample of said patient using oligonucleotide primers specific to a region of said apoE gene common to apoE2, apoE3 and apoE4 alleles; and

b) genotyping said patient's apoE isoforms by DNA sequencing of said amplified DNA of step a) for indirectly determining the number of copies of the apoE gene allele E4 in said biological sample; wherein one or two copies of E4 indicates a level of incidence of late-onset of Alzheimer's disease and a lowered age of death.

14. The method of claim 13, wherein said primers are selected from the sets consisting of:

a) 5' ACAGAATTCGCCCCGGCCTGGTACAC 3';
5' TAAGCTTGGCACGGCTGTCCAAGGA 3'; and

b) 5' ATCAAGCTTTCGCCCGCCCATCCCAGCCCTTC 3';
5' CGTGAATTCGCATGGGCTGCAGGCTTCGGCGTTC 3'.

15. The method of claim 13, wherein said amplifying step a) is effected by polymerase chain reaction technique or by ligase chain reaction technique.

16. The method of claim 13, wherein one copy of apoE4 indicates that said patient has about 70% risk of developing Alzheimer's disease and a mean age of death of about 76.5 years.

17. The method of claim 13, wherein two copies of apoE4 indicates that said patient has about 90% risk of developing Alzheimer's disease and a mean age of death of about 72.1 years.

18. A method of diagnosing or prognosing Alzheimer's disease in a patient, which comprises:

a) amplifying genomic DNA encoding apoE in a biological sample of said patient using oligonucleotide primers specific to a region of said apoE gene common to apoE2, apoE3 and apoE4 alleles; and

b) genotyping said patient's apoE isoforms by DNA sequencing of said amplified DNA of step a) for indirectly determining the number of copies of the apoE gene allele E4 in said biological sample; wherein one or two copies of E4 indicates a level of incidence of late-onset of Alzheimer's disease and a lowered age of death.

19. The method of claim 18, wherein said primers are selected from the sets consisting of:

a) 5' ACAGAATTCGCCCCGGCCTGGTACAC 3';
5' TAAGCTTGGCACGGCTGTCCAAGGA 3'; and

b) 5' ATCAAGCTTTCGCCCGCCCATCCCAGCCCTTC 3';
5' CGTGAATTCGCATGGGCTGCAGGCTTCGGCGTTC 3'.

20. The method of claim 18, wherein said amplifying step a) is effected by polymerase chain reaction technique or by ligase chain reaction technique.

21. The method of claim 18, wherein one copy of apoE4 indicates that said patient has about 70% risk of developing Alzheimer's disease and a mean age of death of about 76.5 years.

22. The method of claim 18, wherein two copies of apoE4 indicates that said patient has about 90% risk of developing Alzheimer's disease and a mean age of death of about 72.1 years.

23. The use of specific apoE primers for the clinical determination of the risk for the late-onset of Alzheimer's disease of a patient, which comprises primers selected from the group consisting of:

D= 5' TACTGCACCAGGCGGCCTCG 3';
E= 5' TACTGCACCAGGCGGCCTCA 3';
F= 5' GCCTGGTACACTGCCAGTCG 3';
G= 5' GCCTGGTACACTGCCAGTCA 3'; and
H= 5' AAGGAGATTGAAGGCCTACAAAT 3'.

24. The use of specific apoE primers for the diagnosis or prognosis of Alzheimer's disease in a patient, which comprises primers selected from the group consisting of:

D= 5' TACTGCACCAGGCGGCCTCG 3';
E= 5' TACTGCACCAGGCGGCCTCA 3';
F= 5' GCCTGGTACACTGCCAGTCG 3';
G= 5' GCCTGGTACACTGCCAGTCA 3'; and
H= 5' AAGGAGATTGAAGGCCTACAAAT 3'.

25. The use of primers specific to a region of said apoE gene common to apoE2, apoE3 and apoE4 alleles for the clinical determination of the risk for the late-onset of Alzheimer's disease of a patient, which comprises primers selected from the sets consisting of:

a) 5' ACAGAATTCGCCCCGGCCTGGTACAC 3';
5' TAAGCTTGGCACGGCTGTCCAAGGA 3'; and

b) 5' ATCAAGCTTTCGCCCGCCCATCCCAGCCCTTC 3';
5' CGTGAATTCGCATGGGCTGCAGGCTTCGGCGTTC 3'.

26. The use of primers specific to a region of said apoE gene common to apoE2, apoE3 and apoE4 alleles for the diagnosis or prognosis of Alzheimer's disease in a patient, which comprises primers selected from the sets consisting of:

a) 5' ACAGAATTCGCCCCGGCCTGGTACAC 3';
5' TAAGCTTGGCACGGCTGTCCAAGGA 3'; and

b) 5' ATCAAGCTTTCGCCCGCCCATCCCAGCCCTTC 3';
5' CGTGAATTCGCATGGGCTGCAGGCTTCGGCGTTC 3'.

	E4	E3	E2
ALLELE FREQUENCY ON CHROMOSOME 19 IN EASTERN CANADA	0.152	0.770	0.078

PROTEIN CODED BY EACH ALLELE (APO E IS 299 AMINOACIDS LONG)	APO E4	APO E3	APO E2
- SITE 112	ARG	CYS	CYS
- SITE 158	ARG	ARG	CYS

PHENOTYPES		RELATIVE CHARGE	%	ISOELECTRIC PROFILE (⊖ ⊕)
HOMOZYGOTES	E4/4	+2	3.9	
	E3/3	+1	61.8	
	E2/2	0	2.0	
HETEROZYGOTES	E4/3		20.6	
	E4/2		9.8	
	E3/2		2.0	

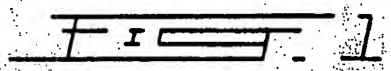

FIG. 1

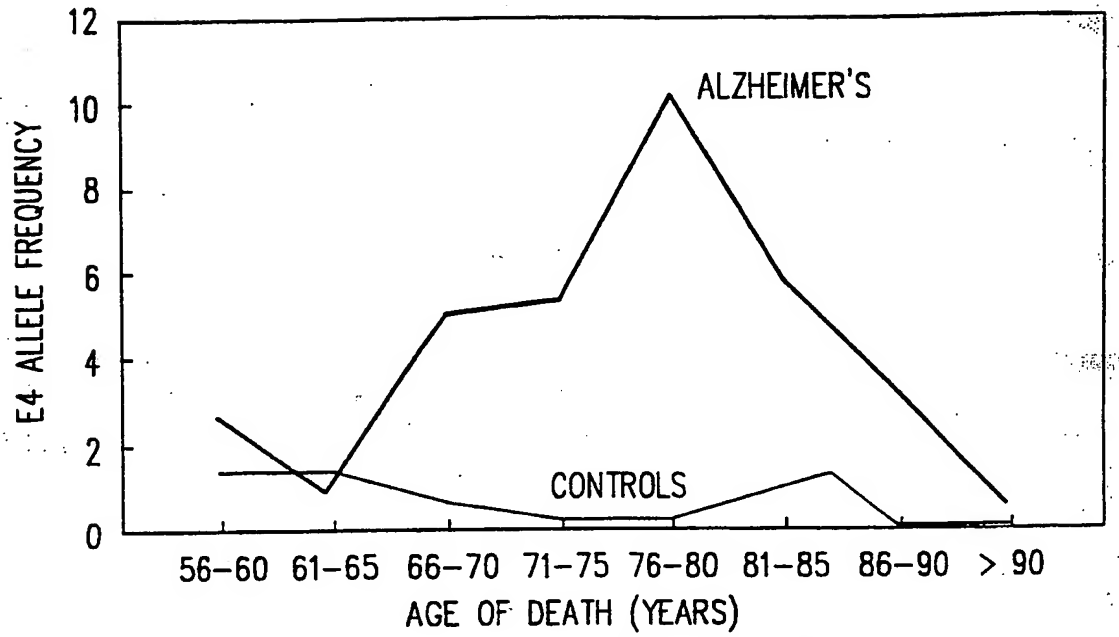
12
10
8
6
4
2
0
E4 ALLELE FREQUENCY
ALZHEIMER'S
CONTROLS
56–60 61–65 66–70 71–75 76–80 81–85 86–90 > 90
AGE OF DEATH (YEARS)

FIG. 2

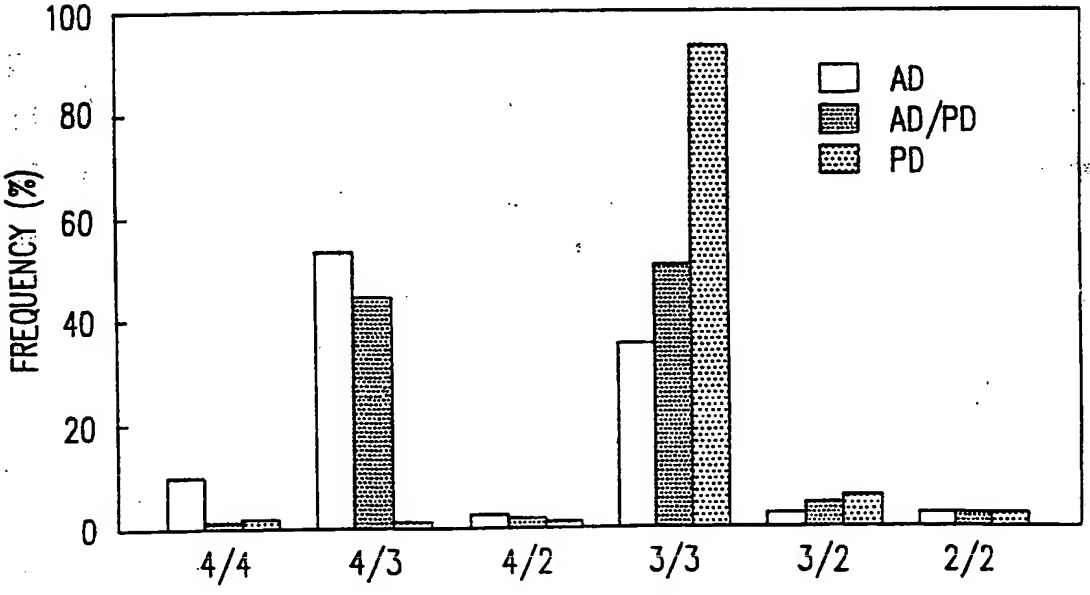
100
80
60
40
20
0
FREQUENCY (%)
AD
AD/PD
PD
4/4 4/3 4/2 3/3 3/2 2/2
Apo E Genotype

FIG. 3

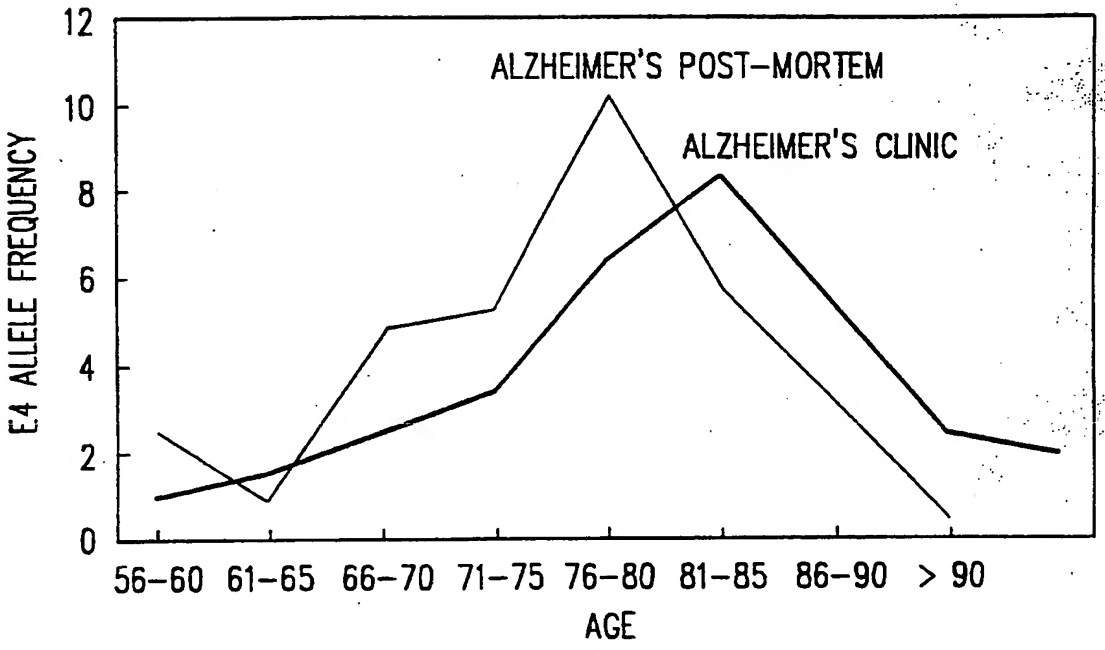
ALZHEIMER'S POST-MORTEM
ALZHEIMER'S CLINIC
E4 ALLELE FREQUENCY
0
2
4
6
8
10
12
56-60
61-65
66-70
71-75
76-80
81-85
86-90
> 90
AGE

FIG. 4

INTERNATIONAL SEARCH REPORT

Intern: al Application No
PCT/CA 94/00681

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LANCET THE, vol.342, 18 September 1993, LONDON GB pages 737 - 738 HARDY, J. ET AL 'apolipoprotein e genotype and alzheimer's disease' see the whole document ---	1,7,13, 18
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.90, March 1993, WASHINGTON US pages 1977 - 1981 STRITTMATTER, W. ET AL 'apolipoprotein e: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial alzheimer disease' see the whole document --- -/--	1,7,13, 18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 1995	02.05.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (- 31-70) 340-3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 94/00681

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE LANCET, vol.342, 18 September 1993, LONDON GB pages 697 - 699 POIRIER, J. ET AL 'apolipoprotein e polymorphism and alzheimer's disease' cited in the application see the whole document	1,7,13, 18
X	THE LANCET, vol.342, 20 November 1993, LONDON GB page 1308 ANWAR, N. ET AL. 'apolipoprotein e4 allele and alzheimer's disease' see the whole document	1,3,7, 13,18
X	THE LANCET, vol.342, 20 November 1993, LONDON GB page 1309 LUCOTTE, G. ET AL 'apolipoprotein e4 allele and alzheimer's disease.' see the whole document	1,3,7, 13,18
A	THE LANCET, vol.337, 11 May 1991, LONDON GB pages 1158 - 1159 WRENHAM, P. ET AL 'apolipoprotein e genotyping by one stage PCR' see the whole document	1-26
T	WO,A,94 09155 (DUKE UNIVERSITY) 28 April 1994 see the whole document	1-26
A	WO,A,91 13075 (ORION-YHTYMÄ OY) 5 September 1991 see page 20 - page 32, line 12	1-26

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern al Application No
PCT/CA 94/00681

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		CN-A-	1092525	21-09-94
		EP-A-	0625212	23-11-94
WO-A-9113075	05-09-91	AU-B-	642709	28-10-93
		AU-A-	7235191	18-09-91
		JP-T-	5504477	15-07-93